

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Pasteurization of human milk by a benchtop High_Temperature Short_Time device

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1590623> since 2020-10-28T12:39:38Z

Published version:

DOI:<http://dx.doi.org/10.1016/j.ifset.2016.07.004>

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in INNOVATIVE FOOD SCIENCE & EMERGING TECHNOLOGIES, 36C, 2016, <http://dx.doi.org/10.1016/j.ifset.2016.07.004>.

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), <http://dx.doi.org/10.1016/j.ifset.2016.07.004>

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/>

Pasteurization of human milk by a benchtop High-Temperature Short-Time device.

Marzia Giribaldi ^{a,b}, Alessandra Coscia ^c, Chiara Peila ^c, Sara Antoniazzi ^a, Cristina Lamberti ^a,
Marco Ortoffi ^d, Guido E. Moro ^e, Enrico Bertino ^c, Tiziana Civera ^d, Laura Cavallarin ^{a*}

^a Institute of Sciences of Food Production, National Research Council, Grugliasco (To), Italy.

^b Food & Nutrition Research Center, Council for Agricultural Research and Economics, Rome,
Italy.

^c Neonatal Intensive Care Unit, Department of Public Health and Pediatric, University of Turin,
Turin, Italy.

^d Department of Veterinary Sciences, University of Turin, Grugliasco (To), Italy.

^e Italian Association of Human Milk Banks, Milan, Italy.

*Corresponding author: Laura Cavallarin, CNR – Institute of Sciences of Food Production, Largo
Braccini 2, 10095 Grugliasco (To), Italy. Telephone: 0039.011.670.92.34; Fax: 0039.011.670.9297;
E-mail: laura.cavallarin@ispa.cnr.it

19 **ABSTRACT**

20 A new small-scale continuous-flow High-Temperature Short-Time (HTST) pasteurizer has been
21 designed for treating human milk. The efficacy of the new HTST device was assessed on inoculated
22 *Listeria monocytogenes*, *Staphylococcus aureus* and *Chronobacter sakazakii*, as well as on raw
23 human milk bacteria. The milk biochemical quality after HTST pasteurization was assessed in
24 comparison to a standard Holder pasteurization, by determining the secretory IgAs (sIgAs) content,
25 the protein profile, lysozyme and the bile salt stimulated lipase (BSSL) activities. No pathogen or
26 bacterial growth was detected after HTST pasteurization with the new instrument. Changes in the
27 protein profile were observed in the milk pasteurized according to both processes. The sIgAs
28 content and BSSL activity were significantly higher in the milk pasteurized with the new device
29 than in the same milk treated by the standard Holder pasteurization. In conclusion, the new HTST
30 apparatus: (i) can effectively pasteurize human milk with a better retention of sIgAs content and
31 BSSL activity; (ii) comply to human milk banking safety requirements.

32

33 **Keywords:** Donor milk; HTST; Bile Salt Stimulated Lipase; Challenge tests; Secretory IgAs.

34

35

36 **INDUSTRIAL RELEVANCE**

37 Currently, 206 active human milk banks are located in Europe (and 13 more are planned). The
38 majority of the European banks still use Holder-based pasteurizers, which, despite efficacy in
39 ensuring microbiological safety, are known to reduce/disrupt important nutritional and non-
40 nutritional biological factors. Although already widely established in food industry, the advantages
41 of HTST technology were tested only at small laboratory scale for human milk. The device tested in
42 the present research was specifically designed to provide human milk banks with the technology
43 they need to ensure a safe and lower-impact pasteurization process, that is suitable for processing
44 different volumes of donations. The device can pasteurize up to 10 l of milk per hour, with a

45 minimum volume of 100 ml. The system is designed to be cleaned-in-place (CIP) after each
46 pasteurization run and sanitized immediately prior to the next use, being thus more suitable for
47 treating pools of milk from different donors than milk from single donations. Italian and EU patents
48 have been filed for the device, within a partnership between public research institutions,
49 stakeholders (Italian association of donor milk banks), and a private company in the sector of dairy
50 processing equipment. The device has achieved a Technology Readiness Level (TRL) 6 (Prototype
51 demonstration in a relevant environment). The cost of the new device will be comparable to that of
52 a typical human milk Holder pasteurizer.

53

54

55 **Abbreviations:**

56 Bile Salt Stimulated Lipase (BSSL)

57 Colon Forming Units (CFU)

58 High-Temperature Short-Time (HTST)

59 Holder pasteurization (HoP)

60 Human milk (HM)

61 Human Milk Bank (HMB)

62 Inoculated human milk (IHM)

63 Original human milk (OHM)

64 Pasteurized human milk (PHM)

65 Raw human milk (RHM)

66 Secretory IgAs (sIgAs)

67 Sterile human milk (SHM)

68 Total Viable Bacteria Count (TVC)

69

70 **Funding**

71 This work was supported by the Fondazione Cassa di Risparmio di Torino [grant number
72 2014.0850]; and the Italian Association of Donor Milk Banks.

73

74 **Conflicts of Interests**

75 LC MG SA EB AC have competing interests since they are the inventors of a pending patent
76 application on the HTST pasteurizer for human milk described in the paper (Patent application no.
77 EP 15176792.8-1358/2014). No conflict of interest exists for the remaining authors.

78

79 1. INTRODUCTION

80 Thermal treatments are commonly applied for food processing, because of their ability to kill
81 pathogens and inactivate potentially detrimental enzymes, such as lipases and proteases. The Holder
82 pasteurization (HoP) method (62.5 °C for 30 min) is currently the recommended pasteurization
83 method for human milk banks (HMBs), as it ensures that the human milk (HM) is microbiologically
84 safe (Arslanoglu et al., 2010). However, heat processing, particularly under severe conditions, may
85 give rise to chemical and physical changes that can impair the organoleptic properties and reduce
86 the content or bioavailability of some nutrients (Arslanoglu et al., 2013; Tully, Jones, & Tully,
87 2001). HM has an elevated biological value, thanks to the casein-to-whey ratio, high essential
88 amino acid concentration and immunological components, such as immunoglobulins, lysozyme,
89 and lactoferrin, which convey relevant antimicrobial properties (Andreas, Kampmann, & Mehring
90 Le-Doare, 2015; Giribaldi, et al. 2012). Thermal treatments may also cause the unfolding of milk
91 fat globule membrane proteins and whey proteins, whose end products are typically associated with
92 off-flavours (Contador, Delgado, García-Parra, Garrido, & Ramírez, 2015). The loss of some
93 biologically active components, including immunological components, due to HoP, is a main limit
94 to the spread of donor human milk (Moro & Arslanoglu, 2012; Tully et al., 2001). The
95 maximization of the biological and nutritional quality of donor HM is considered a scientific and
96 social priority: the ESPGHAN Committee on Nutrition has pointed out that “future research should
97 focus on the improvement of milk processing in human milk banks, particularly of heat treatment”
98 (Arslanoglu et al., 2013).

99 In a previous study by our group (Baro et al., 2011), the HTST method (72 °C for 15 s) showed to
100 better preserve, in comparison to HoP, the milk protein profile and some of the key active
101 components of HM, with potential consequences on the availability of important nutritive
102 compounds, such as fatty acids and available lysine.

103 In previous studies concerning the application of HTST to HM, other non-commercial devices were
104 used, usually at a laboratory scale. Some authors used laboratory equipment, mainly consisting of

105 stainless steel tubing systems submerged in thermostated water baths, through which HM was
106 pumped (Dhar, Fichtali, Skura, Nakai, & Davidson, 1996; Goldsmith, Eitenmiller, Toledo, &
107 Barnhart, 1983; Terpstra et al., 2007); others injected milk through a sterile water stream in a plate-
108 type industrial heat exchanger (Goldblum et al., 1984); some research studies were conducted by
109 directly heating and rotating small aliquots of milk, to simulate the typical thin-layering of dairy
110 industry HTST devices (Goeltz et al., 2009; Hamprecht et al., 2004). Several studies involved
111 simply heating small aliquots (from 40 μ L to 4 mL) of HM in a bulk process (Mayayo et al., 2014;
112 Mayayo et al., 2016; Silvestre et al., 2006; Silvestre et al., 2008). Moreover, variable heating times
113 (5-15 s) and temperatures (71-75 °C) were adopted. All the reported processes, with the exception
114 of that of Goldblum et al. (1984), are substantially different from industrial HTST processes.
115 However, the introduction of HTST into the HMB routine has not been possible to date, due to the
116 lack of specific low-volume designed instrumentation. In most of studies concerning the HTST
117 processing of HM, quantitative comparisons, with respect to standard pasteurization, have been
118 described, but HoP was often simulated on small aliquots, rather than being performed according to
119 real HMB-implemented protocols, thus representing a possible bias for the generalization of the
120 comparison with novel technologies (Peila et al., 2015).

121 When projecting, creating, testing and patenting a new type of low-volume HTST pasteurizer
122 (Cavallarin et al., 2015), it was considered that: i) it was intended for HMBs, and thus consistency
123 with guideline requirements was mandatory; ii) the dairy industry HTST standards (72 °C holding
124 temperature for 15 s holding time) had to be fulfilled; iii) temperature control had to be ensured by
125 means of adequate probes; iv) a comparison with the HoP process had to be made using the HMB
126 implemented device.

127 The present research is aimed at reporting the efficacy of a new, low-volume, continuous flow
128 commercial HTST pasteurizer on HM quality in terms of: (i) bacterial inactivation, and (ii)
129 preservation of the main immunological and nutritional components.

130

131

132 **2. MATERIALS AND METHODS**

133 **2.1 Preliminary tests**

134 In order to verify the pasteurization process, a preliminary experiment was run. A bovine milk
135 sample was pasteurized and the alkaline phosphatase and peroxidase activities were determined in
136 the treated sample. Alkaline phosphatase is a heat sensitive enzyme in milk that is used as indicator
137 of pasteurization. If milk is properly pasteurized, alkaline phosphatase is inactivated.
138 Lactoperoxidase is one of the most heat-stable enzymes found in milk and it is preserved after a
139 correct HTST pasteurization. The raw bovine milk was purchased from a local automatic
140 distributor, collected in a sterile Pyrex bottle, delivered refrigerated to the lab and stored
141 refrigerated until it was processed. The milk (1 L) was divided into two aliquots: one was kept
142 refrigerated until it was analysed; the other was subjected to HTST pasteurization by means of the
143 new instrument (described in 2.3), collected in a sterile Pyrex bottle and stored refrigerated until the
144 analysis. Alkaline phosphatase activity was tested by means of the enzymatic hydrolysis of p-
145 nitrophenol phosphate, which yields p-nitrophenol and inorganic phosphate (ISO/TS 6090, 2004).
146 Peroxidase activity was determined by means of Storch's peroxidase test, which measures the
147 oxygen transfer from hydrogen peroxide to other readily oxidisable substances (Council Directive
148 92/46/EEC).

149

150 **2.2 Sample collection and pooling of specimens**

151 The HM samples were obtained from the HMB of the Città della Scienza e della Salute in Torino,
152 Italy, from eight healthy donor mothers. The donors cleaned their hands and breasts according to
153 the HMB guidelines. The milk specimens were collected in sterile bisphenol-free polypropylene
154 bottles (Flormed, Naples, Italy) using a breast pump and stored, by the HMB, at -20°C until
155 processed.

156 The HM samples were pooled separately for the two experiments, which were referred to as
157 Experiment 1 and Experiment 2. Panel A and panel B in Figure 1 show the experimental workflows
158 for Experiment 1 and Experiment 2, respectively.

159 In Experiment 1, frozen samples from individual HM donors were thawed overnight in a
160 refrigerator, and then in tap water, and were pooled to achieve a final volume of about 2 L in a
161 sterile Pyrex bottle. They were then mixed carefully, divided into 100 mL aliquots and placed in
162 sterile bisphenol-free polypropylene bottles. One aliquot of original HM (OHM) was used directly
163 to determine the HM background microflora. The remaining aliquots were pasteurized intensively
164 in a water bath for 1 hour at 63 °C (prolonged Holder Pasteurization) to kill all of the existing
165 vegetative forms of microorganisms (SHM). One aliquot was kept for about 20 h in the refrigerator,
166 and then analysed to verify the absence of microbial contaminants. The other aliquots were
167 immediately frozen and used later for inoculation (IHM) (section 2.4.3).

168 In Experiment 2, samples from individual HM donors were obtained frozen from the HMB, thawed
169 overnight in a refrigerator, and then in tap water, and were pooled to achieve a final volume of
170 about 400 mL in a sterile Pyrex bottle. They were mixed carefully and divided into three sterile
171 bisphenol-free polypropylene bottles. Two aliquots were subjected to standard HoP, in two separate
172 trays, in the HMB facilities. The two samples were processed in the same batch and differed only for
173 their position in the pasteurizer. One aliquot was subjected to HTST pasteurization using the new
174 instrument. The Raw (RHM), Holder (HoP-HM) and HTST (HTST-HM) pasteurized samples were
175 kept frozen at -20°C until analysis, with the exception of 10 mL *per* sample, which was kept
176 refrigerated for about 20 h before being used for microbial screening.

177

178 **2.3 Pasteurization equipment**

179 HoP was performed in an HM pasteurizer (Metallarredinox, Zingonia-Verdellino (Bg), Italy)
180 located in the HMB of the Città della Scienza e della Salute in Torino, Italy. A patent pending
181 HTST- based proprietary system (Giada s.r.l, Villafranca Piemonte (To), Italy) (Cavallarin et al.,

2015), specifically created for use in HMBs, was used for HM pasteurization at 72 °C for 15 s. The new instrument is a bench-top device that consists of a system of tubular heat exchangers, which are used for both heating and cooling. The thermostatic stay section has been designed to be isolated, and temperature monitoring is achieved by means of specific digital probes placed at critical control points. The new HTST pasteurizer was designed to meet the specific requirements of safety and efficacy of HMBs: an increase in milk temperature in a few seconds, a thermostatic stay of 15 s (max temperature drop of 1.5 °C during pasteurization) and fast cooling of the pasteurized milk (15 s to achieve a lower temperature than 20 °C).

190

191 **2.4 Experiment 1- Challenge tests**

192 *2.4.1 Microbiological profiling of the RHM and OHM background microflora.*

193 In order to assess the natural contamination of the milk, the OHM (Pool 1) and RHM (Pool 2)
194 background microflora were determined in duplicate. This process included enumeration of the total
195 aerobic mesophilic viable count (by Plate Count Agar (Merck, Darmstadt, Germany) – method
196 EN/ISO 4833, 2006), Enterobacteriaceae (by Crystal-Violet Neutral-Red Bile Glucose Agar
197 (Merck) - method AFNOR V08-054) and coagulase-positive *Staphylococci* (by Baird Parker RPF
198 Agar (Biolife Italiana, Milan, Italy) – method EN/ISO 6888, 1999). The same microbiological tests
199 were performed on SHM, to verify the efficacy of the prolonged Holder pasteurization before
200 inoculation.

201

202 *2.4.2 Bacterial test strains and preparation of the cell suspensions.*

203 *L. monocytogenes* (ATCC 7644), *S. aureus* (ATCC 33862) and *C. sakazakii* (ATCC 51329) were
204 used for the inoculation studies. Working stocks of *L. monocytogenes*, *S. aureus*, and *C. sakazakii*
205 were kept at –20 °C in Brain Heart Infusion nutrient broth (Oxoid Limited, Basingstoke, UK).
206 Nutrient broth was added with 20% glycerol as cryogenic protective agent. Fresh cultures were
207 prepared in their early stationary growth phase for each experiment by inoculating a loop of the

208 frozen culture in 9 mL of the respective sterile culture medium and then incubating the cultures at
209 37 °C for 24 h. The microbial counts were monitored by measuring the optical density at 600 nm
210 and confirmed using a culture-based method. The resulting *L.monocytogenes*, *S. aureus* and *C.*
211 *sakazakii* cell suspensions contained about 10^9 colon forming units (CFU)/mL (OD 0.90 ± 0.03).
212 The culture-based method involved spreading 0.1 mL of the appropriate serial dilutions in peptone
213 water (Merck) onto specific appropriate media, incubated at 37 °C, and the colonies were counted
214 after 24 h: final concentration of 1.5×10^9 CFU/mL for *L. monocytogenes*, 3×10^9 CFU/mL for *S.*
215 *aureus* and 1.6×10^9 CFU/mL for *C. sakazakii* were confirmed by means of plate counts on
216 appropriate selective media.

217 2.4.3 SHM inoculum.

218 *L. monocytogenes*, *S. aureus* and *C. sakazakii* were inoculated in 500 mL of SHM in sterile Pyrex
219 bottles (see Table 1 for the inoculum loads). Each pathogen was inoculated in a single batch.
220 Inoculated samples (IHM) were kept refrigerated until they were pasteurized, for a maximum of 30
221 min.

222 2.4.4 HTST pasteurization of IHM and cleaning of the system.

223 IHM samples of each pathogen were HTST pasteurized (PHM) and collected in sterile Pyrex
224 bottles. The samples were kept refrigerated for about 20 h before the microbiological analyses.
225 After each pasteurization cycle, the HTST equipment was cleaned by pumping 3% v/v Divoflow
226 (JohnsonDiversey S.p.A., Milan, Italy) detergent solution at 50 °C (1 L) through the system in the
227 CIP (cleaning-in-place) mode for 10 min, and this was followed by tap water for 5 min. The day
228 after each experiment, rinsing water (500 mL) was allowed to recirculate in the system for 10
229 minutes, collected in a sterile Pyrex bottle and checked for pathogenic growth.

230 2.4.5 Microbiological analyses of the HTST pasteurized IHM.

231 Colony counts of PHM were performed in triplicate. As the absence of pathogen growth is required
232 after pasteurization in HMB, undiluted samples were also subjected to microbiological screening.

233 The presence of *S. aureus* was determined using the method described in 2.4.1. *L. monocytogenes*
234 was evaluated by enriching with Demi-Fraser and Fraser (Sifin, Berlin, Germany) media incubated
235 at 37 °C for 24 h (EN/ISO 11290, 1996); a subsequent growth on OCLA and PALCAM agar
236 mediums (Oxoid) was allowed for both enriching media at 37 °C for 48 h. *C. sakazakii* was
237 determined by measuring the growth on the *Enterobacter sakazakii* Isolation Agar (ESIA) ready-
238 made plates (Liofilchem s.r.l. Roseto degli Abruzzi (Te), Italy) (AFNOR V08-054).

239

240 **2.5 Experiment 2: Evaluation of the biochemical profile of HTST and Holder pasteurized** 241 **milk.**

242 The RHM, HTST-HM and HoP - HM from trays 1 and 2 of the HMB Holder pasteurizer were
243 assayed to establish the sIgAs content, BSSL and lysozyme activity, as well as the protein profile.
244 The sIgAs were assayed on 1:10,000 diluted samples, using an ELISA kit (Biovendor, Kassel,
245 germany) following manufacturer's instructions, in triplicate. BSSL activity was measured in
246 triplicate according to a previously described protocol (Bertino et al., 2013), with minor
247 modifications. Briefly, an aliquot of 2 mL of substrate solution (25 M Tris-HCl pH 9.0, 0.25 mM 2-
248 methoxyethanol, 0.53 mM p-nitrophenyl myristate in DMSO, 5 mM sodium cholate) was added to
249 50 µL of undiluted (for all pasteurized samples) or diluted (1:1000 for RHM) milk samples, mixed
250 and left to incubate at room temperature for 60 min. Then, 1 mL of stop solution (acetone/n-heptane
251 5:2 vol/vol) was added, and immediately mixed by inversion. After centrifugation for 15 min at
252 6500rpm at 4 °C, absorbance of the lower aqueous phase was measured at 405 nm. Lysozyme
253 activity was tested in triplicate on 1:200 diluted samples using a fluorescence-based kit (EnzChek
254 Lysozyme Assay Kit, Thermo Fisher Scientific, Waltham, USA) following to the manufacturer's
255 instructions. The assay measures lysozyme activity as the increase in fluorescence resulting from
256 the enzyme activity on *Micrococcus lysodeikticus* cell walls labeled with fluorescein . Active
257 lysozyme enzyme hydrolyzes the b-(1-4)-glucosidic linkages between the N-acetylmuramic acid
258 and N-acetyl-D-glucosamine residues in the mucopolysaccharide cell wall, relieving the quenching

259 and yielding an increase in fluorescence that is proportional to lysozyme activity. The total protein
260 content was determined in triplicate on HM samples, skimmed by means of centrifugation at 2000g
261 at 4 °C for 30 min, using a 2DQuant kit (GE Healthcare Europe, Milan, Italy), following the
262 manufacturer's instructions. The protein profile (in reducing and non reducing conditions, 5 µg of
263 proteins) was visualized by mono dimensional electrophoresis on a 10-well 12% Nu-PAGE®
264 precast gel, with MES as the running buffer, on a Novex Mini-cell (Thermo Fisher Scientific) at
265 200 V. The gels were stained with Blue Coomassie Colloidal stain, according to the protocol
266 already described in a previous study (Giribaldi et al., 2013).

267

268 **2.6 Statistical analyses**

269 Data on the performed biochemical analyses were analysed using the KyPlot 2.0 statistical software
270 (Kyens Lab Inc., Tokyo, Japan), with one-way ANOVA at a significance of below 0.05 to assess
271 significant differences. When the calculated values of F were significant, Tukey's post-hoc analysis
272 was used to classify any significant difference between the mean values.

273

274 **3. RESULTS**

275 **3.1 Setting up the pasteurization parameters.**

276 The instrument was preliminarily tested on bovine milk in order to assess its suitability for
277 performing a proper HTST pasteurization cycle, and the test was successful. The new HTST system
278 correctly performed a pasteurization cycle (72 °C for 15 s), since the marker enzyme, that is,
279 alkaline phosphatase, resulted to be inactivated in a pasteurized bovine milk sample. A shorter
280 retention time (10 s) was also tested, and was found not to be sufficient to inactivate the alkaline
281 phosphatase in the bovine milk. By setting the pasteurization conditions at 72 °C for 15 s, the
282 lactoperoxidase activity was preserved in a pasteurized bovine milk sample, thus meeting the
283 requirements for the HTST pasteurization of bovine milk.

284

285 **3.2 Microbiological and challenge tests on HTST pasteurized HM.**

286 In order to assess the natural contamination of the starting donated milk pool (OHM) from the
287 HMB, its background microflora was analysed. The total viable bacteria count (TVC), coagulase
288 positive *Stafilococci* and Enterobacteriaceae loads are reported in Table 2. No *L. monocytogenes* or
289 *C. sakazakii* were found.

290 After prolonged pasteurization by means of HoP, the absence of vegetative forms of
291 microorganisms was assessed, and the resulting milk was considered “sterile” (SHM). This milk
292 was used as the starting material for the challenge tests. The milk was inoculated (IHM) with *L.*
293 *monocytogenes*, *S. aureus* and *C. sakazakii* in order to perform the challenge tests; the bacterial
294 counts of the starting inocula are reported in Table 1. PHM did not show any pathogen growth after
295 the HTST pasteurization conducted with the new prototype, in any of the performed challenge tests,
296 as assessed by both qualitative and quantitative methods. At the same time, the rinsing water did not
297 present any growth of the inoculated microorganism. The efficiency of both the Holder and HTST
298 pasteurization was also tested in Experiment 2 (see Figure1B for the experimental design) by
299 measuring the bacterial loads before and after the pasteurization of naturally contaminated milk
300 (Table 1). No bacteria were detected in the milk following either pasteurization procedure.

301

302 **3.3 Evaluation of the biochemical profile of HTST and Holder pasteurized milk.**

303 The qualitative changes between RHM, HTST-HM and HoP-HM, as performed directly in an HMB
304 device, were determined in Experiment 2 by measuring the sIgAs content, and BSSL and lysozyme
305 activity, and by profiling the protein profile in both reducing and non-reducing conditions.

306 A monodimensional denaturing protein electrophoresis was run on all the samples, in order to
307 profile any major change induced by the different pasteurization methods on the protein pattern.
308 The resulting images are shown in Figure 2. The protein profiles seemed to be very similar, as far as
309 the reducing conditions are used (lanes 1-4), without any visible degradation of the specific bands
310 following any of the pasteurization processes. Accordingly, the lysozyme activity was measured

and no significant difference was found between HTST- and HoP-HM (Tab. 3). In addition, the lysozyme activity value measured in both of the pasteurized samples did not differ significantly from the value found in RHM (50.2 ± 0.2 U/ μ L). However, the protein profiles in the non-reducing conditions (lanes 6-9) displayed some differences in the high molecular weight bands following all the pasteurization processes.

The sIgAs content of HTST- and HoP-HM was determined and retention rates with respect to RHM were calculated. HoP-HM was found on average to retain 46.3% sIgAs, although a noteworthy difference was observed between the two samples pasteurized in the two different trays ($36.8 \pm 21.6\%$ vs. $55.8 \pm 3.1\%$). HTST-HM showed a significantly higher sIgAs retention. As for the BSSL activity retention, in both HoP and HTST the enzymatic activity resulted to be almost completely destroyed with respect to RHM (145 ± 22 μ mol/mL/min); nevertheless, HTST-HM displayed a significantly higher activity than HoP-HM (Tab. 3). No significant difference in HoP-HM was observed between the two trays (data not shown).

324

325 4. DISCUSSION

A new apparatus for the HTST pasteurization of HM has been tested in order to verify its ability to eliminate selected pathogens as well as to assess the effect of pasteurization on the milk protein profile and selected nutritional and immunological parameters. The instrument was preliminarily tested to assess its ability to perform a proper HTST pasteurization cycle on bovine milk, and some commonly used technological parameters confirmed that the test was successful (positive peroxidase and negative alkaline phosphatase reactions in the pasteurized milk). The raw human milk used for the experiment resulted to be highly contaminated by coagulase-positive *Staphylococcus* spp. Human milk contains commensal bacteria, and in particular non-pathogenic coagulase-negative *Staphylococci* from the bacterial flora of the maternal skin. The transmission of pathogens, such as *Staphylococcus aureus*, has also been reported (Heikkila & Saris, 2003). The starting raw milk pools delivered from the bank showed a similar background microflora to that

337 already observed in a previous experiment by our group on HM from a Neonatal Intensive Care
338 Unit (Giribaldi et al., 2013).

339 The milk was completely pasteurized by prolonged Holder treatment, thus constituting SHM for
340 inoculation. The inocula for the challenge tests (*L. monocytogenes*, *S. aureus* and *C. sakazakii*) were
341 chosen on the grounds of being high-risk pathogens that cause the contamination of milk and
342 neonatal infections. All the pathogens were eliminated by the HTST pasteurization performed with
343 the new instrument. When HoP and HTST were used to eradicate the natural background microflora
344 of the HM in the present study, no bacterial growth was observed, even in the presence of high
345 microbial loads, including Enterobacteriaceae. These results are in agreement with the first reports
346 that measured the efficacy of HTST on naturally contaminated HM: Goldblum et al. (1984) found
347 complete pasteurization of HM after HTST by using a plate-type industrial heat exchanger and
348 injecting HM in a sterile water stream. Challenge tests by Dhar et al. (1996) and by Terpstra et al.
349 (2007) confirmed the efficacy of small-scale laboratory devices, based on continuous flow HTST
350 pasteurization, on inoculated *Escherichia coli*, *S. aureus* and *Streptococcus agalactiae*. Terpstra et
351 al. (2007) also investigated the efficacy of HTST on HM samples inoculated with 3 lipid-enveloped
352 and 2 non-enveloped viruses. The system was shown to be highly effective against lipid-enveloped
353 viruses (HIV and marker viruses for hepatitis B and C). The efficacy of a low cost simulated HTST
354 to be used in resource-limited settings (FoneAstra system) has also been reported. The FoneAstra
355 system resulted to be a reliable, low-cost pasteurization monitoring and reporting systems to be
356 used worldwide for treating donor milk (Naicker et al., 2015)

357 The biochemical parameters used to assess the quality of pasteurized milk were selected from
358 among the most commonly measured in previous studies on the effect of HoP on HM. Attention
359 was focused in particular on parameters that are significant from an immunological and/or
360 nutritional point-of-view, and which had previously been reported to be decreased by HoP
361 pasteurization (Tully et al., 2001). Both sIgAs and BSSL resulted to be significantly higher in the
362 milk pasteurized with the new HTST pasteurizer than in the milk treated with the standard HoP,

363 conducted according to the HMBs guidelines and using an HMB pasteurizer. sIgAs represents the
364 majority of IgA in HM (Goldsmith et al., 1983). In early reports, the retention of sIgAs and /or the
365 total IgAs following HTST was found to be complete (Goldblum et al., 1984), decreased with
366 respect to raw milk (about 60% for Dhar et al., 1996) and equal to that of HoP with respect to raw
367 milk (63% according to Goldsmith et al., 1983; 80% for Hamprecht et al., 2004; 40% for Mayayo et
368 al., 2016). In the present experiment, the amount of HTST-retained IgAs following the treatment of
369 the milk with the new apparatus was almost twice as high as the IgAs content of the same milk
370 processed with HoP (as measured in a real HMB implemented pasteurizer), and higher than the
371 levels reported in previous investigations. However, the results concerning the IgAs content
372 following the two pasteurization systems need to be confirmed on a larger number of samples, since
373 it is well documented that the range of variation of IgAs values as affected by Holder pasteurization
374 is very high (Peila et al., submitted). As for lysozyme activity, no significant difference was found in
375 the present study in either of pasteurized samples, compared to raw milk. This result is in contrast
376 with previous findings, which reported a decrease in lysozyme as an effect of HoP (Sousa, Santos,
377 Fidalgo, Delgadillo, & Saraiva, 2014; Viazis, Farkas, & Allen, 2007), as well as of HTST
378 pasteurization (Mayayo et al., 2016). In all these reports, lysozyme activity was determined using a
379 *Micrococcus lysodeikticus*-based turbidimetric assay, which measures to what extent bacterial
380 growth is prevented by the addition of lysozyme-containing samples. However, HM is a complex
381 mixture of several anti-bacterial factors, whose degradation may also have an impact on the lower
382 rates of the measured “lysozyme activity”, when it is measured as previously described. In the
383 present study, an attempt was made to overcome this problem using an assay in which the specific
384 lysozyme muramidase activity is measured by quantifying the fluorescence released from labeled
385 *M. lysodeikticus* cell walls. This difference in the analytical approach can explain the discordance
386 from literature and points out the need for appropriate analytical tools and a correct interpretation of
387 the results.

388 Following both types of pasteurization, BSSL displayed an activity almost 1,000-fold lower in
389 comparison to the activity recorded in the RHM sample. The almost complete disruption of lipase
390 activity following any pasteurization treatment, confirms previous reports (Goldblum et al., 1984;
391 Hamprecht et al. 2004), although the measured activity in those investigations was not exclusively
392 due to BSSL. The lipase activity in the present study has been much lower than that observed in a
393 previous study by our group (Baro et al., 2011). Again in that case, lipase activity was measured
394 without the induction of bile salts. Nevertheless, the BSSL bioactivity resulted to be significantly
395 higher in the HTST treated samples than in the HoP samples.

396 As far as the protein profile modification in pasteurized HM is concerned, no qualitative difference
397 was found between RHM, HoP and HTST in the reducing conditions. This result is in contrast with
398 previous findings of our group (Baro et al., 2011) and of Mayayo et al. (2014) for HoP, which were
399 obtained in non-reducing electrophoretic conditions. Conformational differences, if any, may be
400 detected more easily when non-reducing electrophoresis is used. This is confirmed by the fact that
401 Mayayo et al. (2014) were not able to profile any change in the protein pattern following HoP when
402 reducing conditions were used. Therefore, a non-reducing electrophoresis was run, and some slight
403 degree of polymerization for high molecular weight protein bands was observed (probably
404 ascribable to lactoferrin and immunoglobulins), thus confirming the results of Baro et al. (2011) and
405 Mayayo et al. (2014). In conclusion, the new bench-top HTST apparatus can effectively pasteurize
406 HM with a better retention of the sIgG content and BSSL activity, compared to standard Holder
407 pasteurization.

408 REFERENCES

- 409 1. AFNOR. (2009). V08-054. Dénombrement des entérobactéries présumées par comptage des
410 colonies à 30 °C ou à 37 °C. In: *Microbiologie des aliments*.
- 411 2. Andreas, N. J., Kampmann, B., & Mehring Le-Doare, K. (2015). Human breast milk: A review
412 on its composition and bioactivity. *Early Human Development*, 91(11), 629-635.
- 413 3. Arslanoglu, S., Bertino, E., Tonetto, P., De Nisi, G., Ambrozzi, A.M., Biasini, A., ... Moro, G.
414 E. (2010). Guidelines for the establishment and operation of a donor human milk bank. *Journal*
415 *of Maternal-Fetal and Neonatal Medicine*, 23(S2),1–20.
- 416 4. Arslanoglu, S., Corpeleijn, W., Moro, G. E., Braegger, C., Campoy, C., Colomb, V., ... Van
417 Goudoever, J. (2013). Donor human milk for preterm infants: Current evidence and research
418 directions. *Journal of Pediatric Gastroenterology and Nutrition*, 57(4), 535-542.
- 419 5. Baro, C., Giribaldi, M., Arslanoglu, S., Giuffrida, M. G., Dellavalle, G., Conti, A., ... Bertino,
420 E. (2011). Effect of two pasteurization methods on the protein content of human milk.
421 *Frontiers in Bioscience - Elite*, 3E(3), 818-829.
- 422 6. Bertino, E., Giribaldi, M., Baro, C., Giancotti, V., Pazzi, M., Peila, C., ... Gastaldi, D. (2013).
423 Effect of prolonged refrigeration on the lipid profile, lipase activity, and oxidative status of
424 human milk. *Journal of Pediatric Gastroenterology and Nutrition*, 56(4), 390-396.
- 425 7. Cavallarin, L., Bertino, E., Giribaldi, M., Antoniazzi, S., Coscia, A, Gariglio, G. M. (2015).
426 Continuous flow pasteurizer for small amounts of liquid food. Patent application: EP
427 15176792.8-1358– Patent holders: CNR, Università di Torino, Giada s.a.s.
- 428 8. Contador, R., Delgado, F. J., García-Parra, J., Garrido, M., & Ramírez, R. (2015). Volatile
429 profile of breast milk subjected to high-pressure processing or thermal treatment. *Food*
430 *Chemistry*, 180, 17-24.
- 431 9. Council Directive 92/46/EEC. (1992). Laying down the health rules for the production and
432 placing on the market of raw milk, heat-treated milk and milk-based products.

- 433 10. Dhar, J., Fichtali, J., Skura, B. J., Nakai, S., & Davidson, A. G. F. (1996). Pasteurization
434 efficiency of a HTST system for human milk. *Journal of Food Science*, 61(3), 569-572.
- 435 11. EN/ISO 11290. (1996). *Microbiology of food and animal feeding stuffs. Horizontal method for*
436 *the detection and enumeration of Listeria monocytogenes - Part 1: detection method*. Geneva:
437 International Organization for Standardization.
- 438 12. EN/ISO 6888. (1999). *Microbiology of food and animal feeding stuffs. Horizontal method for*
439 *the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other*
440 *species)*. Geneva: International Organization for Standardization.
- 441 13. EN/ISO:4833. (2003). *Microbiology of food and animal feeding stuffs. Horizontal method for*
442 *the enumeration of microorganisms. Colony-count technique at 30°C*. Geneva: International
443 Organization for Standardization.
- 444 14. Giribaldi, M., Cavallarin, L., Baro, C., Di Nicola, P., Coscia, A., & Bertino, E. (2012).
445 Biological and nutritional aspects of human milk in feeding of preterm infants. *Food and*
446 *Nutritional Science*, 3(12), 1682-1687.
- 447 15. Giribaldi, M., Ortoffi, M. F., Giuffrida, M. G., Gastaldi, D., Peila, C., Coscia, A., ... Bertino, E.
448 (2013). Effect of prolonged refrigeration on the protein and microbial profile of human milk.
449 *International Dairy Journal*, 31(2), 121-126.
- 450 16. Goelz, R., Hihn, E., Hamprecht, K., Dietz, K., Jahn, G., Poets, C., & Elmlinger, M. (2009).
451 Effects of different CMV-heat-inactivation-methods on growth factors in human breast milk.
452 *Pediatric Research*, 65(4), 458-461.
- 453 17. Goldblum, R. M., Dill, C. W., Albrecht, T. B., Alford, E. S., Garza, C., & Goldman, A. S.
454 (1984). Rapid high-temperature treatment of human milk. *The Journal of Pediatrics*, 104(3),
455 380-385.
- 456 18. Goldsmith, S. J., Eitenmiller, I. R. R., Toledo, R. T., & Barnhart, H. M. (1983). Effects of
457 processing and storage on the water-soluble vitamin content of human milk. *Journal of Food*
458 *Science*, 48(3), 994-995.

- 459 19. Hamprecht, K., Maschmann, J., Müller, D., Dietz, K., Besenthal, I., Goelz, R., . . . Jahn, G.
460 (2004). Cytomegalovirus (CMV) inactivation in breast milk: Reassessment of pasteurization
461 and freeze-thawing. *Pediatric Research*, 56(4), 529-535.
- 462 20. Heikkilä, M. P., & Saris, P. E. J. (2003). Inhibition of *Staphylococcus aureus* by the
463 commensal bacteria of human milk. *Journal of Applied Microbiology*, 95(3), 471-478.
- 464 21. ISO/TS 6090. (2004). *Milk and dried milk, buttermilk and buttermilk powder, whey and whey*
465 *powder. Detection of phosphatase activity*. Geneva: International Organization for
466 Standardization.
- 467 22. Mayayo, C., Montserrat, M., Ramos, S. J., Martínez-Lorenzo, M. J., Calvo, M., Sánchez, L., &
468 Pérez, M. D. (2014). Kinetic parameters for high-pressure-induced denaturation of lactoferrin
469 in human milk. *International Dairy Journal*, 39(2), 246-252.
- 470 23. Mayayo, C., Montserrat, M., Ramos, S. J., Martínez-Lorenzo, M. J., Calvo, M., Sánchez, L., &
471 Pérez, M. D. (2016). Effect of high pressure and heat treatments on IgA immunoreactivity and
472 lysozyme activity in human milk. *European Food Research and Technology*, 242(6), 891-898.
- 473 24. Moro, G. E., & Arslanoglu, S. (2012). Heat treatment of human milk. *Journal of Pediatric*
474 *Gastroenterology and Nutrition*, 54(2), 165-166.
- 475 25. Naicker, M., Coutsooudis, A., Israel-Ballard, K., Chaudhri, R., Perin, N., Mlisana, K. (2015).
476 Demonstrating the efficacy of the FoneAstra pasteurization monitor for human milk
477 pasteurization in resource-limited settings. *Breastfeeding Medicine* 10(2), 107–112.
- 478 26. Peila, C., Moro, G. E., Bertino, E., Cavallarin, L., Giribaldi, M., Giuliani, F., Cresi, F., Coscia,
479 A. Holder Pasteurization and Donor Human Milk: Effect on Nutrients and Biologically-Active
480 Components. Submitted to: *Nutrients*.
- 481 27. Peila, C., Coscia, A., Bertino, E., Cavallarin, L., Giribaldi, M., & Moro, G. E. (2015). Donor
482 human milk and its nutritional properties: What should we believe? *Journal of Human*
483 *Lactation*, 32(2), 390-391.

- 484 28. Silvestre, D., Ferrer, E., Gayá, J., Jareño, E., Miranda, M., Muriach, M., & Romero, F. J.
485 (2006). Available lysine content in human milk: Stability during manipulation prior to
486 ingestion. *BioFactors*, 26(1), 71-79.
- 487 29. Silvestre, D., Miranda, M., Muriach, M., Almansa, I., Jareno, E., & Romero, F. J. (2008).
488 Antioxidant capacity of human milk: Effect of thermal conditions for the pasteurization. *Acta*
489 *Paediatrica, International Journal of Paediatrics*, 97(8), 1070-1074.
- 490 30. Sousa, S. G., Santos, M. D., Fidalgo, L. G., Delgadillo, I., & Saraiva, J. A. (2014). Effect of
491 thermal pasteurisation and high-pressure processing on immunoglobulin content and lysozyme
492 and lactoperoxidase activity in human colostrum. *Food Chemistry*, 151, 79-85
- 493 31. Terpstra, F. G., Rechtman, D. J., Lee, M. L., Van Hoeij, K., Berg, H., Van Engelenberg, F. A.
494 C., & Van't Wout, A. B. (2007). Antimicrobial and antiviral effect of high-temperature short-
495 time (HTST) pasteurization applied to human milk. *Breastfeeding Medicine*, 2(1), 27-33.
- 496 32. Tully, D.B., Jones, F., & Tully, M.R. (2001). Donor milk: what's in it and what's not. *Journal*
497 *of Human Lactation*, 17(2), 152-155.
- 498 33. Viazis, S., Farkas, B. E., & Allen, J. C. (2007). Effects of high-pressure processing on
499 immunoglobulin A and lysozyme activity in human milk. *Journal of Human Lactation*, 23(3),
500 253-261.

503 **TABLE 1. Challenge tests: effect of HTST pasteurization on human milk.**

Inoculated pathogens	Initial loads in IHM ¹ (CFU/mL)	Final loads in PHM ¹ (CFU/mL)
<i>Listeria monocytogenes</i>	1.2 x 10 ⁶	absent in 25 ml
<i>Staphylococcus aureus</i>	3.0 x 10 ⁶	< 100
<i>Chronobacter sakazakii</i>	1.6 x 10 ⁶	absent in 10 ml

504 ¹ IHM = inoculated human milk; PHM = HTST pasteurized milk.

505

506 **TABLE 2. Background microflora of human milk (starting Pools 1 and 2)**

	Pool 1 (OHM) ¹ (CFU/mL)	Pool 2 (RHM) ¹ (CFU/mL)
Total viable count	1.0 x 10 ⁶	7.7 x 10 ⁴
Coagulase-positive <i>Staphylococci</i>	2.5 x 10 ⁵	1.1 x 10 ⁴
<i>Enterobacteriaceae</i>	< 10	2.1 x 10 ²

507 ¹ OHM = original human milk for Experiment 1; RHM = raw human milk for Experiment 2

508

509 **TABLE 3. Nutritional milk characteristics as affected by Holder and HTST pasteurization.**

Parameter (Unit)	HoP-HM ¹	HTST-HM ¹
sIGAs (% of RHM ¹)	46.3±13.2 ^a	78.9±2.4 ^b
BSSL activity (μmol/min/mL)	0.09±0.03 ^a	0.26±0.10 ^b
Lysozyme activity (U/μL)	52.3±3.2	48.8±0.8

510 ¹ HoP-HM = Holder pasteurized milk; HTST-HM = HTST pasteurized milk; RHM = raw milk

511 ^aThe data represent the standard deviations of the means obtained from three independent replicate
512 experiments. Different superscript letters indicate significant Tukey's post-hoc mean differences
513 across each row.

514

515

Figure Captions

FIGURE 1. Experimental design and workflow for Experiment 1 (Panel A) and Experiment 2 (Panel B).

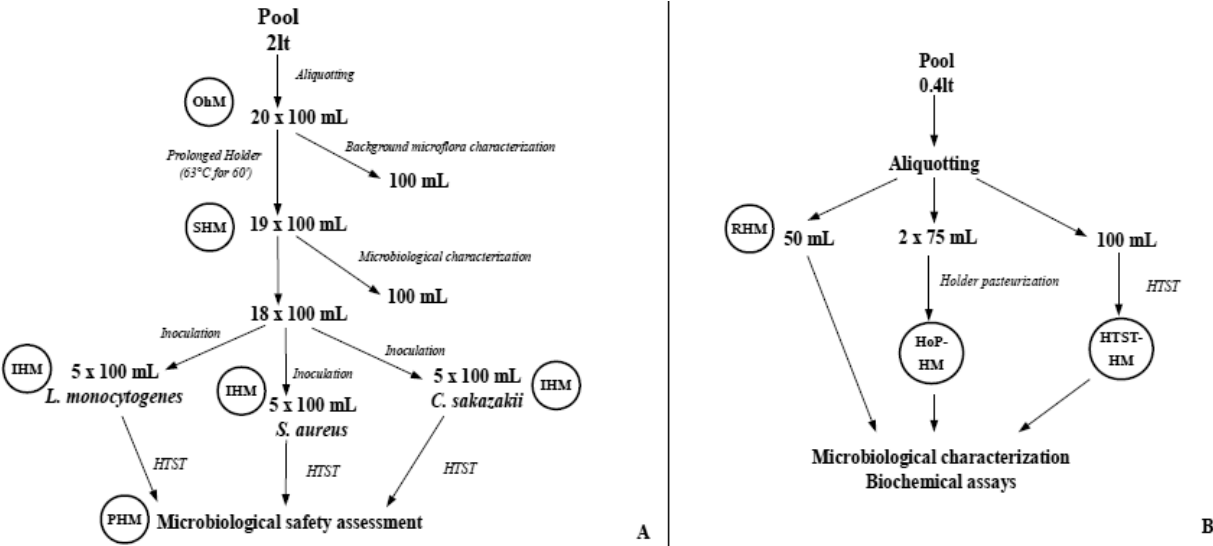
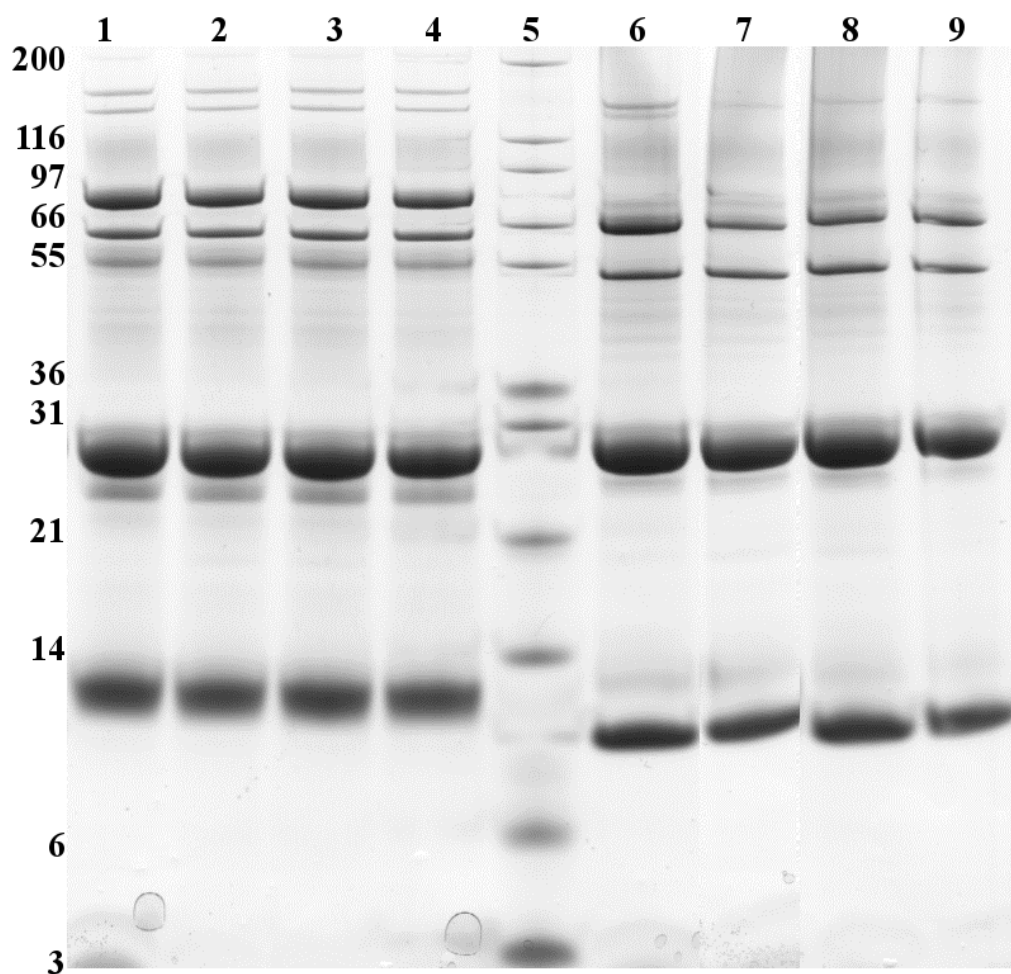


FIGURE 2. NuPAGE protein profile of the HM samples treated with the different pasteurization processes. Lanes 1 to 4, samples run in reducing conditions: 1) RHM = raw milk; 2) HoP-HM = Holder pasteurized milk Tray 1; 3) HoP-HM = Holder pasteurized milk Tray 2; 4) HTST-HM = HTST pasteurized milk; 5) Molecular weight standards. Lanes 6 to 9, samples run in non-reducing conditions: 6) RHM = raw milk; 7) HoP-HM = Holder pasteurized milk Tray 1; 8) HoP-HM = Holder pasteurized milk Tray 2; 9) HTST-HM = HTST pasteurized milk. Colloidal Coomassie Blue stain.



530